

Clumping of *Staphylococcus aureus* in the Peritoneal Cavity of Mice

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ABSTRACT

KAPRAL, FRANK A. (Philadelphia General Hospital, Philadelphia, Pa.). Clumping of *Staphylococcus aureus* in the peritoneal cavity of mice. J. Bacteriol. 92:1188-1195. 1966.—Nonencapsulated strains of *Staphylococcus aureus* inoculated into the peritoneal cavity of mice are promptly clumped by the interaction of fibrinogen with the bound coagulase present on the bacterial surface. Some of the pre-existing leukocytes adhere to the staphylococcal clumps during the 1st hr, but phagocytosis is minimal. During the 2nd hr, there is an influx of neutrophils into the region, and these form a thick layer around the staphylococcal clumps and, apparently, prevent further egress of toxin. Leukocytes in proximity to the organisms undergo degeneration, but cells located externally maintain an effective barrier and, thus, confine the organisms. The encapsulated Smith strain of *S. aureus* is not clumped under these circumstances, presumably because the capsule prevents the bound coagulase-fibrinogen interaction.

Earlier observations (4, 5; Kapral, Postepy Mikrobiol., *in press*) revealed that certain strains of *Staphylococcus aureus*, when inoculated into the peritoneal cavity of mice or rabbits, were quickly clumped. Since this phenomenon appeared to be of some importance in the pathogenesis of staphylococcal peritonitis, a more detailed study of the clumping reaction was made.

MATERIALS AND METHODS

Strains. *S. aureus* 18Z (6) is a conventional strain possessing both bound and soluble coagulase, as well as hyaluronidase, fibrinolysin, α hemolysin, and δ hemolysin. The mutant 18Z-B definitely lacks bound coagulase and is not simply covered over by a capsule.

S. aureus P78 (7) is similar to the 18Z strain, except that it is capable of producing greater amounts of α toxin.

S. aureus Smith mucoid (diffuse), described by Hunt and Moses (3), possesses a permanent capsule (9) which prevents the demonstration of the bound coagulase. *S. aureus* Smith nonmucoid (compact) lacks the capsule, and the presence of the bound coagulase is readily demonstrable.

Animal inoculation. Organisms grown in Trypticase Soy Broth (BBL) were washed with saline containing 1% Trypticase Soy Broth. This latter addition prevented the rapid loss in viability, commonly seen when staphylococci are placed in saline; yet, the con-

centration of broth was not sufficient to permit multiplication for at least 6 hr. Suspensions were diluted to the desired concentration and either were used immediately or were placed in ampoules, quick frozen, and stored in Dry Ice until needed. No demonstrable loss in viability of frozen suspensions occurred, even after 6 months of storage. Frozen preparations were thawed immediately before use and were diluted to the desired concentration with saline containing 1% Trypticase Soy Broth.

White Swiss male mice, weighing 25 to 30 g, were used throughout. Mice were inoculated intraperitoneally with the desired dose in a volume of 0.2 ml. At various times after inoculation, organisms were recovered from the peritoneal cavity by sacrificing the mouse and washing out the peritoneal cavity with five 3- to 5-ml samples of saline containing 1% Trypticase Soy Broth. Plate counts were done on the pooled washings, and the total culturable units were calculated. The remainder of the washings was passed through a 0.45- μ membrane filter (Millipore Filter Corp., Bedford, Mass.) and drained barely to dryness. The membrane was then fixed in 95% ethyl alcohol and was allowed to dry. These membranes were subsequently stained with Giemsa, were decolorized with methanol, were cleared with xylol, and were mounted in resin.

In other instances, clumps of staphylococci were individually removed from the peritoneal cavity, were fixed in neutral buffered formaldehyde, embedded, sectioned, stained with hematoxylin-eosin, and mounted in resin.

Leukocytes in peritoneal cavity. The staphylococcal suspension was developed under conditions where

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CO₂ was either added or excluded during growth. Previous studies (Kapral and Meyer, Bacteriol. Proc., p. 44, 1966) have indicated that prior growth in the presence of CO₂ markedly affects the ability of organisms to produce α toxin subsequent to inoculation into the peritoneal cavity; this, in turn, influences the virulence of the staphylococci in this route of infection. Grown under such conditions, equivalent doses of these types of inocula would lead to either 100% mortality (cocci grown in presence of 20% CO₂) or more than 90% survival (cocci grown in complete absence of CO₂).

Groups of mice were inoculated intraperitoneally with 0.2 ml of the desired staphylococcal suspension. At various intervals after inoculation, the mice were sacrificed (or used immediately upon death), and the peritoneal cavity was washed out with five 2-ml samples of heparin-treated (1:20,000) saline. The volume of the pooled washings was measured, the leukocytes were counted in a hemocytometer, and the total number of cells was calculated.

Fibrinogen levels. Fibrinogen present in plasma or in peritoneal fluid was measured indirectly by the method of Duthie (2), in which standardized suspensions of *S. aureus* 18Z (final concentrations 5×10^9 per milliliter) were added to serial dilutions of the body fluids. After agitation in a reciprocating shaker for various intervals, the degree of clumping was read as zero to 4+ with 45 times magnification. The relative activity among different preparations was determined by comparing similar end points (2+) of the titrations.

RESULTS

The dose-response curve for *S. aureus* 18Z, grown in Trypticase Soy Broth and inoculated intraperitoneally into adult mice, shows the LD₁₀ at 3×10^8 to 4×10^8 , the LD₅₀ at 9×10^8 , and the LD₉₀ at 2×10^9 (5). No deaths ever occurred with doses of 2×10^8 or less. With this in mind, we inoculated mice with doses of 18Z corresponding to the LD₅₀, as well as with doses which either would be nonlethal (10^8 to 2×10^8) or would result in essentially 100% mortality (2×10^9 to 3×10^9). In mice given the higher doses (8×10^8 , 2×10^9), there was a rapid clumping of the organisms in the peritoneal cavity, as reflected by a reduction of the number of culturable units which could be recovered (Fig. 1). This clumping was maximal as early as 8 to 10 min after inoculation. The number of culturable units then remained essentially constant for the next 2 hr. This clumping process was also demonstrable by the examination of the washings collected on membrane filters (Millipore Filter Corp.).

When an initial inoculum of 10^8 cocci (non-lethal dose) was introduced into the peritoneal cavity, there was no evidence of a prompt clumping but rather a continuous decrease in the population. Examination of the washings on mem-

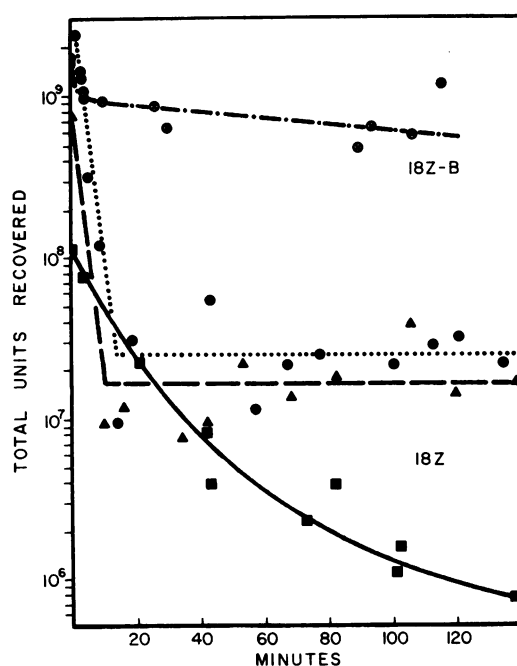


FIG. 1. Number of culturable units recovered from the peritoneal cavity of mice after inoculation of *Staphylococcus* 18Z (three dosage levels) and the bound coagulase-negative mutant 18Z-B.

brane filters failed to reveal any clumping of the organisms.

The inoculation of the mutant 18Z-B (lacking bound coagulase), even in high doses, failed to exhibit any clumping, although a reduction in the number of organisms over a 2-hr period did occur.

When similar studies were performed with *S. aureus* Smith, the mucoid (diffuse) variant, even in high doses, would not clump but actually increased in numbers over a 2-hr period (Fig. 2). This was consistent with the observations of others (1, 8). In contrast, the nonmucoid (compact) variant behaved in the same manner as did the 18Z strain. With the nonmucoid variant in doses of 5×10^8 or greater, prompt clumping occurred, and the number of culturable units thereafter remained essentially constant. The final concentration of culturable units obtained with the nonmucoid Smith was comparable to that previously obtained with the 18Z strain after maximal clumping. Examination of washings collected on membrane filters confirmed that clumping had occurred.

With a dose of 10^8 nonmucoid organisms of the Smith strain, clumping was not evident, but a progressive decrease in the recoverable popula-

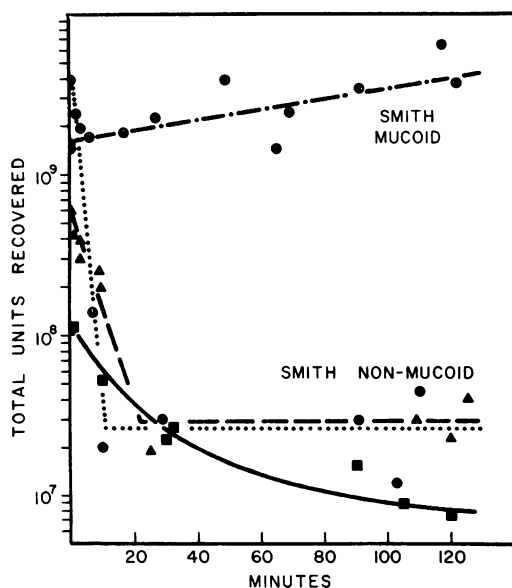


FIG. 2. Number of culturable units recovered from the peritoneal cavity of mice after inoculation of the mucoid *Staphylococcus aureus* Smith and the nonencapsulated mutant (three dosage levels).

tion over the 2-hr period was found. Examination of washings collected on membrane filters confirmed the lack of clumping.

An examination of washings collected on membrane filters (Fig. 3) demonstrated that the clumps of staphylococci (18Z), during the initial periods of observation, were associated with relatively few leukocytes (predominantly monocytes). Figures 3A and 3B show small clumps of staphylococci obtained 8 min after inoculation. A few leukocytes are found on the periphery of these clumps. This situation does not change significantly during the next hr. Figures 3C and 3D illustrate clumps obtained after the organisms had been in the peritoneal cavity for 70 min. Again, comparatively few leukocytes are present on the periphery of the clumps. To permit better visualization of adhering leukocytes, only small clumps are shown in Fig. 3. Most staphylococcal clumps were much larger than these illustrated.

During the 2nd hr after the inoculation of staphylococci into the peritoneal cavity, there was a marked influx of leukocytes into this region; this response was essentially the same regardless of the capacity of the inoculum to produce α toxin after infection (Fig. 4A, 4B). Furthermore, the rapid influx of leukocytes was essentially complete by the 3rd hr.

Mice given the α toxin-induced organisms

(cocci previously grown under 20% CO_2) did not survive beyond 6 hr after infection (Fig. 4A); leukocyte counts from animals dying from infection did not differ significantly from cell counts obtained from mice sacrificed 3 hr after injection. On the other hand, mice inoculated with the same dose of noninduced organisms (cocci previously grown in the complete absence of CO_2) were, for the most part, capable of surviving this infection (Fig. 4B). Leukocyte populations in the peritoneal cavity of mice infected in this manner did show a slow continuous increase from 3 hr until ca. 12 hr after infection (not included in Fig. 4B), when counts averaged 7×10^6 cells per mouse. Thereafter, counts remained stationary for another 12 hr, then slowly returned to normal over the next 3 to 4 days. A few mice in this group did succumb to infection 5 to 8 hr after inoculation, but cell counts from these animals did not differ significantly from those obtained from mice sacrificed during the same interval. Therefore, differences in the ability to produce α toxin after infection did not appear to influence the extent of the leukocyte response, nor did cell counts on animals dead from infection differ from those sacrificed at the same time after inoculation.

The apparent reduction in the leukocyte population observed during the first 10 min after infection seems to be due to the adherence of pre-existing leukocytes to the staphylococcal clumps forming during this period. Such adhering leukocytes were, for the most part, not included in the cell counts, because of poor visualization.

An examination of staphylococcal clumps removed from the peritoneal cavity after the cell response had occurred revealed that these leukocytes formed a thick layer surrounding the clumped organisms. This is illustrated in Fig. 5A, which is a histological section from a clump removed from the peritoneal cavity 3 hr after infection. A prominent leukocyte layer consisting primarily of neutrophils, but with some basophils, became evident around the clumped staphylococci. The leukocytes in this outer layer appeared intact and free from phagocytized staphylococci. A portion of this leukocyte layer under higher magnification is shown in Fig. 5B. In the central region of the clump, the cocci were found together with a considerable amount of cellular debris. This region under higher magnification is illustrated in Fig. 5C. Between and adjacent to the staphylococci, a considerable amount of eosinophilic material was found, together with nuclear remains in different stages of degeneration.

Further observations demonstrated that several

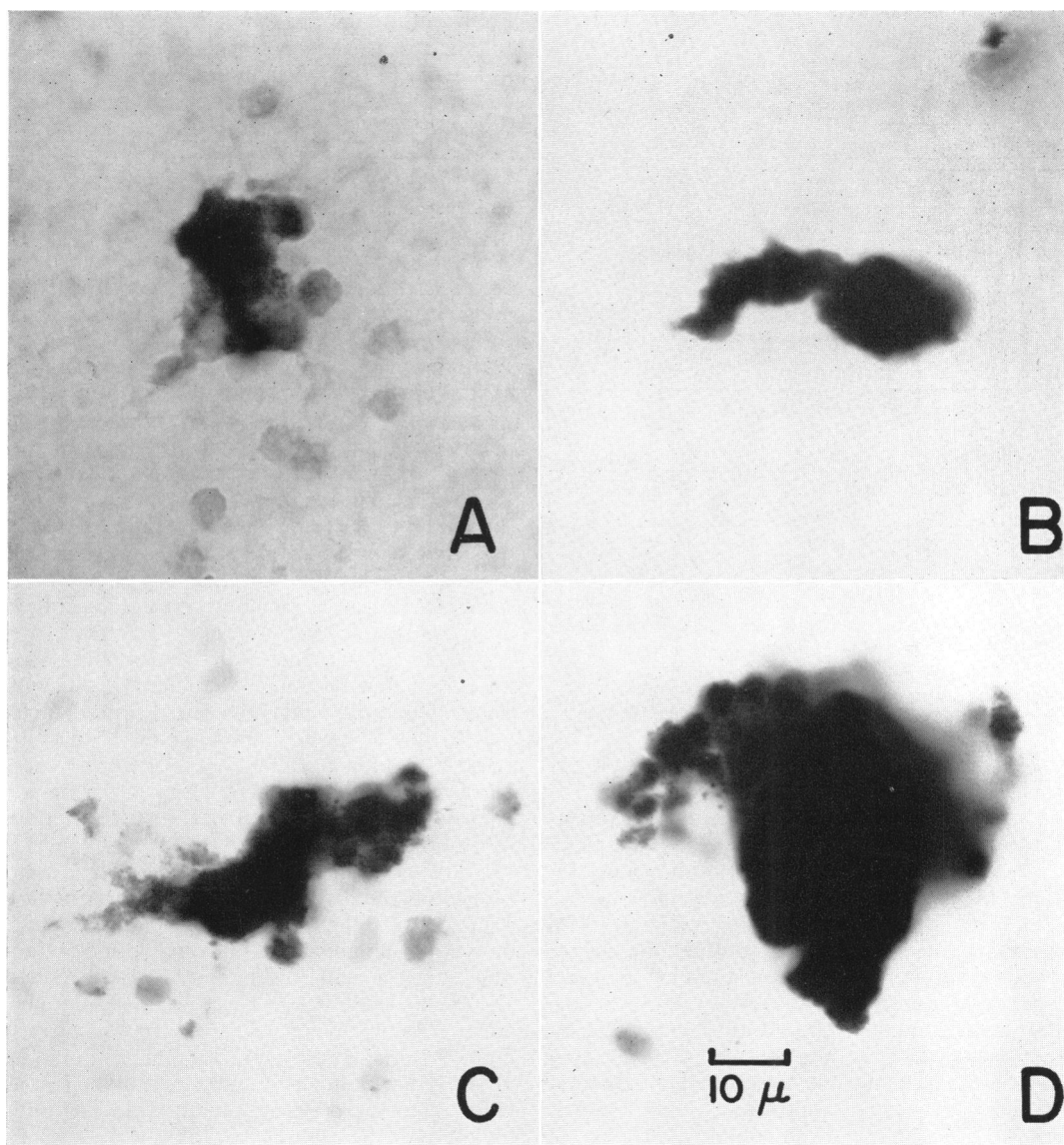


FIG. 3. Clumps of *Staphylococcus aureus* 18Z obtained from the peritoneal cavity of mice, 8 min (3A, 3B) and 70 min (3C, 3D) after inoculation with 3×10^9 organisms. Same magnification used for all micrographs.

clumps of staphylococci, already surrounded by leukocytes, then were aggregated into still larger units, so that almost all the clumps were in one or two very large clusters at the time of death. Figure 5D is a section through one of these large clusters removed at the time of death, which in this particular animal occurred 5 hr after inoculation.

Since clumping within the peritoneal cavity appeared to result from the interaction of fibrinogen with the bound coagulase present on the

surface of the organism, we decided to investigate whether the conditions existing in vivo caused this phenomenon. Duthie (2) has previously shown that the rate and extent of clumping depends upon both the concentration of organisms and the concentration of fibrinogen. Therefore, the ability of *S. aureus* 18Z to clump in mouse plasma was investigated. Heparin-treated plasma was obtained from normal mice, was diluted in saline, and was mixed with washed suspensions of *S. aureus* 18Z; the extent of

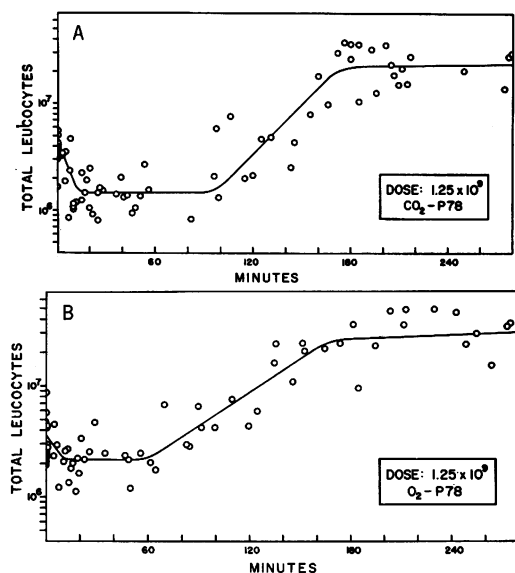


FIG. 4. (A) Total number of leukocytes recovered from the peritoneal cavity of mice after inoculation of 1.25×10^9 α toxin-induced *Staphylococcus aureus* P78 (CO₂-P78). (B) Total number of leukocytes recovered from the peritoneal cavity of mice after inoculation of 1.25×10^9 α toxin-noninduced *S. aureus* P78 (O₂-P78).

clumping was noted (Table 1). In greater concentrations of mouse plasma, "no clumping" and "4+ clumping" occurred with concentrations of organisms corresponding to 2×10^8 and 3×10^9 per milliliter, respectively. With greater dilutions of mouse plasma, the degree of clumping was less or clumping failed to occur. The data illustrated in Table 1 represent the degree of clumping recorded after 30 min. However, with plasma diluted 1:10, the maximal degree of clumping was already attained within 3 min; with plasma diluted 1:40, clumping was maximal by 10 min. Controls in which mouse serum was used instead of plasma did not exhibit any clumping by the organisms.

To determine how the concentration of fibrinogen in the peritoneal fluid compared with that found in the plasma, normal mice were first bled to obtain plasma and then were sacrificed, and some peritoneal fluid was removed. With the method described by Duthie (2), the relative concentration of fibrinogen in these fluids was compared for each animal by adding standardized suspensions of *S. aureus* 18Z to serial twofold dilutions of both the mouse plasma and peritoneal fluid. Clumping was allowed to proceed for 15 min, and the end point (2+ clumping) was determined. It was found that the

fibrinogen in the peritoneal fluid was one-third to one-seventh (mean, one-fourth) that found in the plasma. This indicated that the concentration of fibrinogen in the peritoneal cavity was quite sufficient to insure maximal clumping rates.

Earlier studies have indicated that α toxin was responsible for death with this type of staphylococcal infection (4); yet, from the findings presented here, it seemed obvious that the interval when α toxin could be elaborated by the cocci and subsequently could be assimilated from the peritoneal cavity was limited in duration. Once the clumped organisms were surrounded by the thick leukocyte layer, as seen after 3 hr, it was not likely that any further quantities of α toxin could effectively penetrate the leukocyte barrier and become available for assimilation by the mouse. This contention was supported by the findings that the external portion of the leukocyte layer consisted of cells which appeared intact and uninjured, whereas cells in proximity to the organisms were undergoing degeneration (Fig. 5). Therefore, it appeared likely that, after 3 hr, any toxin produced by the organisms remained confined within the clumps. Furthermore, this implied that, if a lethal dose of toxin was to be produced, then it would have to be elaborated before the clumped organisms were covered by leukocytes (before 3 hr), even though the animal would not necessarily succumb until some hours later.

If this were so, it should be possible to show protection resulting from antitoxin given within a period of 3 hr after infection, whereas little protection should result from antitoxin administered beyond this period. The above reasoning, however, is also based on the knowledge that: (i) antitoxin can effectively protect against intraperitoneal challenge with nonencapsulated strains; (ii) antitoxin rapidly enters the circulation from the peritoneal cavity; and (iii) antitoxin rapidly neutralizes free toxin (4, 5; Kapral, Postepy Mikrobiol., *in press*).

To support the above hypothesis, groups of mice were inoculated intraperitoneally with α toxin-induced *S. aureus* P78. At various times thereafter, subgroups were given 60 units of commercial staphylococcal antitoxin (Connaught Laboratories, Toronto, Canada) by the intraperitoneal route. This dose of antitoxin is capable of giving 100% protection when administered before intraperitoneal challenge (4). Control groups, infected but receiving only saline thereafter, were also included. The results are illustrated in Fig. 6. It can be seen that, with a LD₅₀ dose (1.25×10^9 cocci), good protection resulted if antitoxin was given 2 hr after infection,

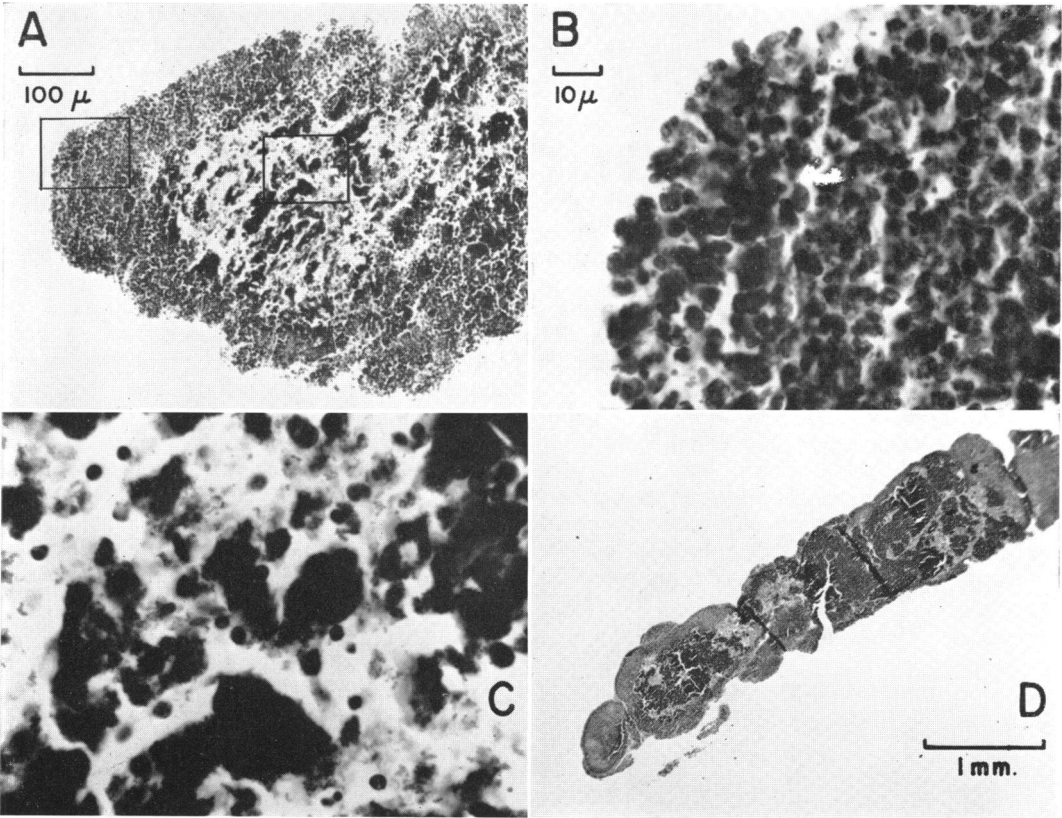


FIG. 5. Histological sections of clumps removed from the peritoneal cavity of mice after the inoculation of 3×10^9 *Staphylococcus aureus* 18Z. (A) Clump removed 3 hr after inoculation. (B and C) Magnified views of the respective areas delineated in A. (D) Cluster of clumps removed after 5 hr in peritoneal cavity.

TABLE 1. Clumping of *Staphylococcus aureus* 18Z in mouse plasma

Final plasma dilution	Final <i>S. aureus</i> per ml				
	3.2×10^9	1.6×10^9	8×10^8	4×10^8	2×10^8
1:4	4+	3+	2+	1+	0
1:40	4+	3+	2+	1+	0
1:160	2+	1+	0	0	0
1:640	0	0	0	0	0

but this was not the case if antitoxin was given at 4 hr. All deaths resulting from this dose of organisms occurred during the period 5 to 20 hr after infection.

With increasing challenge doses, the period for which one could delay antitoxin administration and still achieve protection decreased markedly. This was to be expected, since larger inocula could elaborate a lethal dose of α toxin within a short time after infection and the subsequent

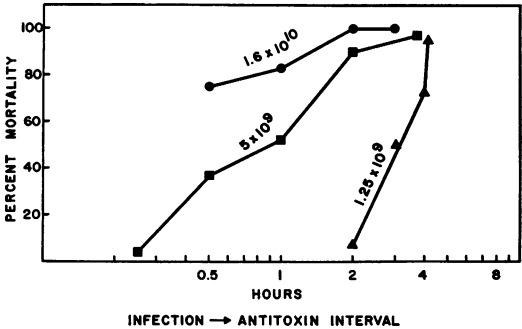


FIG. 6. Degree of protection afforded by 60 units of staphylococcal antitoxin (Connaught Laboratories) when administered intraperitoneally at varying times after infection with *Staphylococcus aureus* P78.

neutralization of any additional toxin would be inconsequential.

The above results were substantiated by studies measuring actual toxin production within the

peritoneal cavity and indicating that toxin was released for about 3 hr before being curtailed. These studies will be reported in detail elsewhere.

DISCUSSION

The observation that conventional strains of *S. aureus* clump after intraperitoneal inoculation has been noted previously, but the findings reported here have necessitated a revision of some details occurring in this type of infection. Furthermore, these data offer a reasonable answer to certain questions heretofore inexplicable (4). A summary of our current concept of events occurring in staphylococcal peritonitis is presented in Fig. 7.

Small doses of nonencapsulated, α toxin-producing staphylococci were not effectively clumped within the peritoneal cavity and were phagocytized by the available leukocytes. Furthermore, the amount of α toxin produced was insufficient for death. With larger doses, the prompt clumping (occurring if the organisms possessed

the bound coagulase) protected the inoculum from the leukocytes except for a minimal amount of phagocytosis proceeding at the periphery. Most of the organisms were, thus, free to elaborate α toxin, which is rapidly assimilated from the peritoneal cavity. However, as soon as the leukocyte influx occurred, the clumped organisms were surrounded by an extensive layer of these cells, thereby preventing further egress of toxin. If a lethal dose of toxin was already assimilated, then the animal would succumb later. If an insufficient quantity of toxin was assimilated, the animal, of course, survived, and the cocci and products remained confined within the clumps. The eventual fate of these organisms will be reported elsewhere.

When encapsulated strains were employed (such as the Smith mucoid), the presence of the capsule prevented the clumping reaction from occurring, but at the same time hindered phagocytosis to a significant degree. If doses were sufficiently small, the available leukocytes could

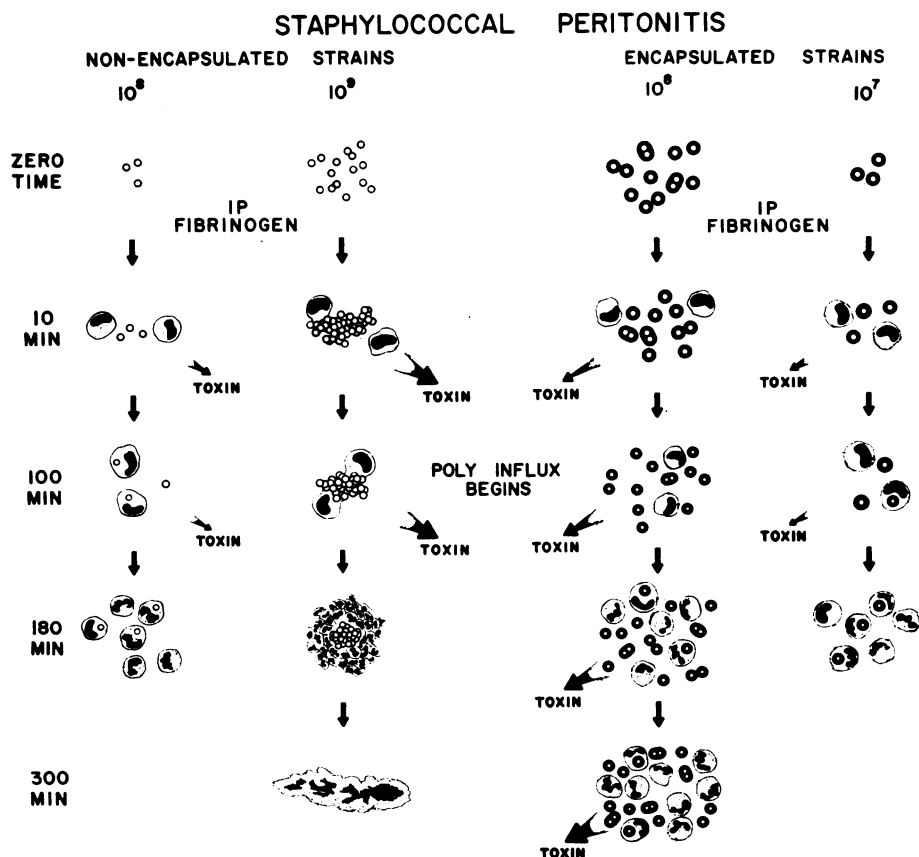


FIG. 7. Summary of events after the intraperitoneal inoculation of mice with *Staphylococcus aureus*.

remove the inoculum before a lethal dose of toxin was produced. However, with larger doses, the organisms could multiply faster than they were phagocytized and could elaborate toxin continuously.

The above explanation can account for the marked differences seen in the effectiveness of antitoxin when used in conjunction with conventional and encapsulated strains (4). After intraperitoneal infection with nonencapsulated strains, the period during which α toxin was available for assimilation was definitely limited by the layering of leukocytes about the organisms; therefore, reasonable doses of antitoxin given early enough could manifest excellent protection. On the other hand, infection with the same dose of an encapsulated strain permitted the sustained production of toxin. Antitoxin, even in very high doses, merely increased the survival time, but death eventually ensued. It is reasonable to assume that, in this case, antitoxin combined with the α toxin as it was produced, until eventually all antitoxin was consumed. Further production of α toxin then proved fatal.

The findings can also explain observed differences in the nature of staphylococcal clumps with time. Freshly clumped staphylococci were readily dispersed by agitation or pipetting, but, after some time in the peritoneal cavity, the clumps were no longer dispersed easily. These differences probably were due to the layer of leukocytes which developed after a few hours and which tended to maintain the integrity of the clumps during agitation.

It is perhaps worth noting that the range of staphylococcal concentrations necessary for 0 to 4+ clumping by mouse plasma (2×10^8 to 3×10^9 per milliliter respectively) coincides quite closely with those required for 0 to 100% mortality after intraperitoneal infection. At present, it is not possible to say whether this is coincidental or whether the concentrations required for clumping set the lower limits for the dose-response relationship. It is known from other studies that differences in the degree to which the inoculum is induced with respect to

α toxin production can shift the entire dose-response curve. These shifts are such that the slopes remain parallel, and the LD_{50} may be increased as much as 20-fold. The nonencapsulated strains used in this study have never been lethal by this route in doses of 10^8 or less. It is likely that the inability of fibrinogen to clump such low concentrations of *S. aureus* permits prompt phagocytosis and precludes the production of a lethal dose of α toxin.

ACKNOWLEDGMENT

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